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Use of a High-resolution Melting Assay to Evaluate HIV Gag Region Diversity in HIV-infected Adults with Different Stages of HIV Infection

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Background: We developed a high-resolution melting assay for HIV diversity, and used the assay to compare the level of HIV *gag* region diversity in adults with different stages of HIV infection.

Methods: The HIV *gag* region was amplified using LightScanner Master Mix amplification buffer (Idaho Technologies, Inc.). The *gag* amplicons were analyzed on a LightScanner instrument to produce a melting curve (–d[fluorescence]/d[temperature]) to determine the high-resolution melting score (the number of degrees over which melting occurred). High-resolution melting *gag* amplicons from 9 mother–infant pairs were cloned and sequenced for assay validation (50 clones/mother; 20 clones/infant). The high-resolution melting assay was then used to analyze samples from 198 adults from the US, including 20 with acute HIV infection, 106 with recent HIV infection (collected a median of 187 days after a negative HIV test, range 14 to 540 days), 38 with chronic HIV infection (infected >2 years, CD4 cell count >50), and 34 with AIDS (CD4 cell count <50). Median high-resolution melting scores were compared using the Wilcoxon rank test.

Results: The median high-resolution melting score for infants (4.3, range 4.2 to 5.3) was higher than that for control plasmids (3.4, range 3.2 to 3.8, P <0.0001), and lower than that for mothers (5.7, range 4.4 to 7.7, P =0.005, exact Wilcoxon rank sum test). The reproducibility of the HRM assay was high (intra-class correlation coefficient: 94%, 95%CI 89% to 98%). High high-resolution melting scores were associated with high genetic diversity (P =0.0002), complexity (P =0.0094), and Shannon entropy (P =0.022). The median (range) high-resolution melting scores for adults were: acute=3.9 (3.5 to 5.4), recent=4.2 (3.5 to 7.5), chronic=5.1 (3.3 to 9.9), AIDS=6.3 (3.8 to 10.1). The median high-resolution melting score in adults with acute HIV infection was lower than in adults with recent HIV infection (P =0.0091), and the median high-resolution melting score in adults with recent HIV infection was lower than in adults with chronic HIV infection or AIDS (P <0.0001). The median HRM score was lower in adults with chronic HIV infection than in adults with AIDS, but the difference was not statistically significant (P =0.061). A high-resolution melting score of >6.3 (mean +3 standard deviations for recent samples) was highly correlated with chronic HIV infection or AIDS (P <0.0001 Fisher's exact test).

Conclusions: The high-resolution melting assay can be used for rapid, high-throughput measurement of HIV diversity without sequencing. In this study, HIV *gag* region diversity increased as HIV infection progressed from acute HIV infection, to recent HIV infection, to chronic HIV infection and AIDS.

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Introduction

HIV diversity is usually studied by analyzing sequences from individual HIV variants. We developed a novel High Resolution Melting (HRM) assay to analyze HIV diversity without sequencing.

Methods

Template Preparation

PCR products used as templates in the HRM assay were generated using the ViroSeq HIV-1 Genotyping System (Celera, Alameda, CA) according to the manufacturer's instructions. ViroSeq PCR products were further diluted 1:10 to yield a concentration of ≈5 ng for use in the HRM assay.

HRM Assay

A region of HIV *gag* was amplified using the LightScanner Master Mix amplification buffer (Idaho Technology, Salt Lake City, UT). The resulting amplicons (~150-235 base pairs) were then analyzed on a LightScanner instrument (Model HR 96). The resulting data were processed to produce a melting curve for each sample representing the negative derivative of the data (-d[fluorescence]/d[temperature]). The left and right margins of each melting curve were marked at the positions at which the curve reached an angle of 30° relative to a horizontal baseline. The HRM score is defined as the difference (number of degrees) between the two borders.

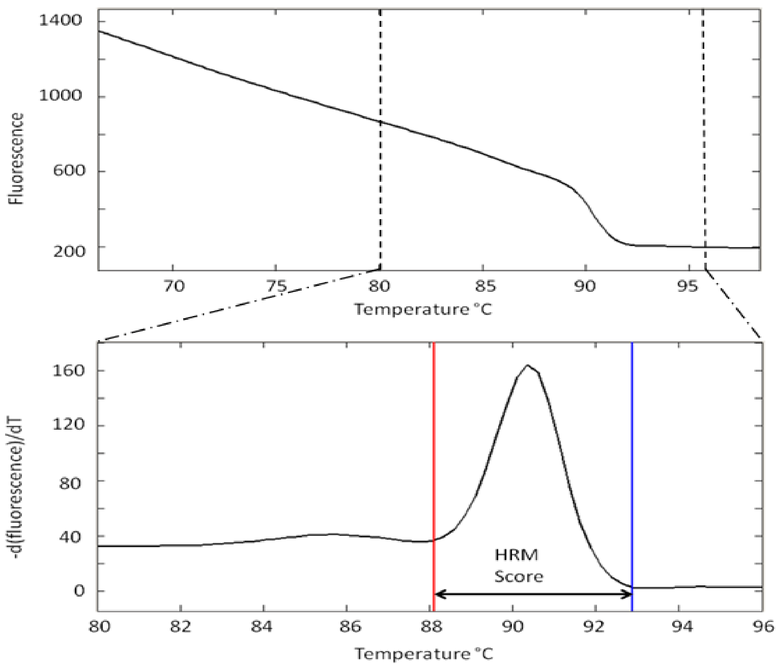

Figure 1. Analysis of HRM data to produce the HRM score.

Results

I. Proof of Concept

We first used the HRM assay to analyze plasmid controls and plasma samples from HIV-infected women and infants (9 mother-infant pairs). The median HRM score for infants (4.3, range 4.2-5.3) was higher than that for control plasmids (3.4, range 3.2-3.8, p<0.001), and lower than that for mothers (5.7, range 4.4-7.7, p=0.005, exact Wilcoxon rank sum test). The melting curves for each mother-infant pair are shown below (Figure 2).

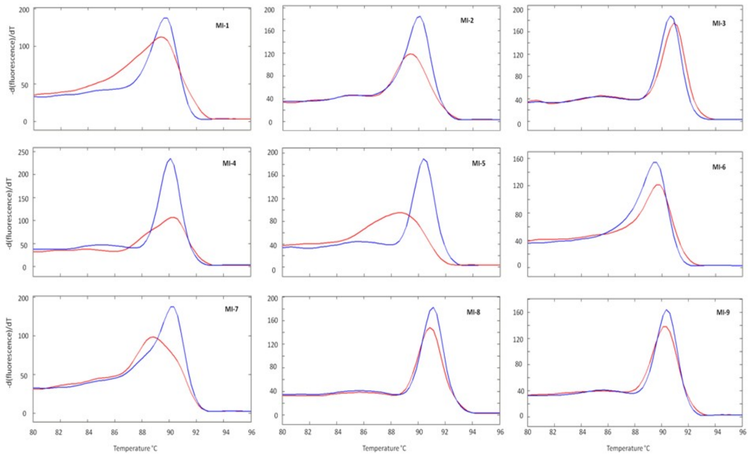

Figure 2. HRM data from HIV-infected women (red) and their infants (blue).

II. Assay Reproducibility

The same samples were analyzed with the HRM assay four times over a one year period. The intra-class correlation coefficient was 94% (95% CI: 89%-98%), indicating a high level of assay reproducibility.

III. Comparison of the HRM Assay to Traditional Sequence-based Diversity Measures

We compared HRM scores to traditional sequence-based measures of genetic diversity. For these studies, the HRM products from the 9 mother-infant pairs were cloned and sequenced (50 clones/woman, 20 clones/infant). Sequences were analyzed using MegaAlign, ClustalX2 and MEGA. Higher HRM scores were associated with higher genetic diversity (p<0.001), higher complexity (p<0.009), and higher Shannon entropy (p<0.022). HRM scores were not associated with length variation (p<0.111).

IV. Biologic Relevance

We compared the HRM scores of adults with different stages of HIV disease (Figure 3).

- Acute (RNA positive, antibody negative, EXPLORE Study, n=20)
- Recent (median 187 days after last HIV negative test, EXPLORE Study, n=106)
- Chronic (HIV pos >2 years, CD4 cell count >50, JHU Moore Clinic and ED, n=38)
- AIDS (CD4 cell count <50, JHU Moore Clinic and JHU ED, n=34)

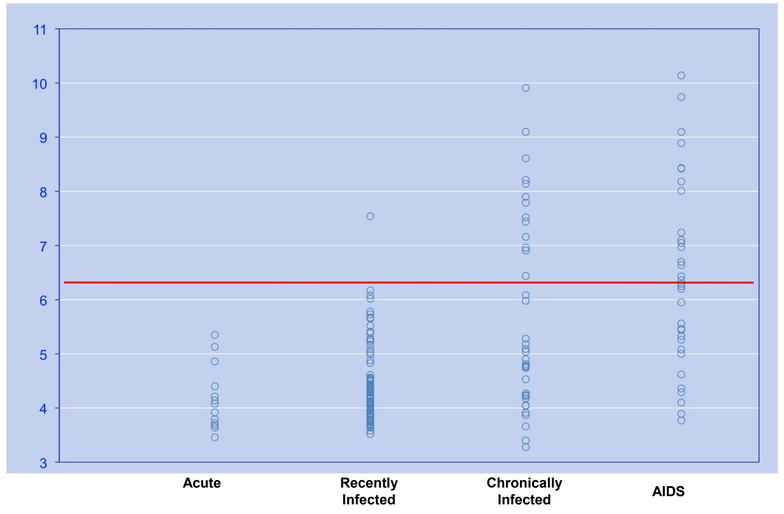

Figure 3. HRM scores from adults with different stages of HIV disease. The red line indicates an HRM score of 6.3 (mean + 3 SD of HRM scores for recently infected adults).

The median HRM score was lower in adults with acute HIV infection than in adults with recent HIV infection (p=0.0091), and was lower in adults with recent HIV infection than in adults with chronic HIV infection and AIDS (p<0.0001). The median HRM score was also lower in adults with chronic HIV infection than in adults with AIDS, but the difference was not statistically significant (p=0.061). An HRM score of >6.3 (mean + 3 SD for samples from adults with recent HIV infection) was associated with chronic HIV infection or AIDS (p<0.0001, Fisher's exact test).

Conclusions

The HRM assay provides a novel, rapid method for assessing HIV diversity without sequencing. In this study, HIV *gag* region diversity increased as HIV infection progressed from acute HIV infection, to recent HIV infection, to chronic HIV infection and AIDS. This assay could be applied to any region of the HIV genome or to other genetic systems that exhibit DNA diversity.

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